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Coagulation assays and plasma fibrinogen concentrations in real-world patients with atrial fibrillation treated with dabigatran

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WHAT IS ALREADY KNOWN ABOUT THIS SUBJECT

- The impact of dabigatran on coagulation assays has been examined mainly using data from healthy volunteers.
- The dilute thrombin time (dTT) is highly correlated with plasma dabigatran concentrations ($r^2 > 0.90$).
- The dTT involves the dilution of test plasma with normal pooled plasma, which is thought to minimize the variability in fibrinogen concentrations.

WHAT THIS STUDY ADDS

- We corroborate the previously reported r^2 values of the screening coagulation assays for plasma dabigatran concentrations in real-world patients on dabigatran.
- We show that plasma fibrinogen concentrations contribute to the variability in TT, which helps to explain the high r^2 of the dTT for plasma dabigatran concentrations.

AIMS

In patients with atrial fibrillation prescribed dabigatran, the aim was to examine the correlation between plasma dabigatran concentrations and the three screening coagulation assays [international normalized ratio (INR), activated partial thromboplastin time (aPTT) and thrombin time (TT)] as well as the dilute thrombin time (dTT) and to examine the contribution of plasma fibrinogen concentrations to the variability in TT results.

METHODS

Plasma from patients with atrial fibrillation on dabigatran were analysed for clotting times and concentrations of fibrinogen and dabigatran. Correlation plots (and associated r^2 values) were generated using these data. The variability in TT results explained by fibrinogen concentrations was quantified using linear regression.

RESULTS

Fifty-two patients (38–94 years old) contributed 120 samples, with plasma dabigatran concentrations ranging from 9 to 408 μg l^{-1}. The r^2 values of INR, aPTT, TT and dTT against plasma dabigatran concentrations were 0.49, 0.54, 0.70 and 0.95, respectively. Plasma fibrinogen concentrations explained some of the residual variability in TT values after taking plasma dabigatran concentrations into account ($r^2 = 0.12$, $P = 0.02$).

CONCLUSIONS

Of the screening coagulation assays, the TT correlated best with plasma dabigatran concentrations. Variability in fibrinogen concentrations accounts for some of the variability in the TT.

Introduction

Dabigatran is a direct reversible thrombin inhibitor that has become established as an alternative to warfarin for anticoagulation in the setting of atrial fibrillation (AF) [1–6]. Some authors recommend that routine laboratory monitoring of coagulation is not required for patients treated with dabigatran [7–10]; however, there is increasing appreciation that laboratory monitoring of coagulation is desirable, at least in specific settings, such as guiding management in the setting of an acute thromboembolic or haemorrhagic event [7–9, 11, 12]. Most of the published reports examining the impact of plasma dabigatran concentrations on coagulation assay results have either used data from plasma spiked with dabigatran (*in vitro*) or have been from healthy volunteers administered dabigatran [7, 13–18]. There is also an emerging body of literature consisting of reports using *ex vivo* data from patients treated with dabigatran, outside of drug-development studies [19–22].

Of the assays that have been examined, the dilute thrombin time (dTT) is often highlighted as the best coagulation assay for assessing individuals treated with dabigatran, because it has a high correlation with plasma dabigatran concentrations ($r^2 > 0.90$) [7, 16, 17, 19, 20, 22]. A commercialized example of the dTT is the Hemoclot® Thrombin Inhibitor assay (HTI; Hyphen BioMed, Neuville-sur-Oise, France). The thrombin time (TT) assay involves the addition of exogenous thrombin to test plasma and measurement of the clotting time. The HTI has an additional step involving an eightfold dilution of the test plasma in saline followed by a further twofold dilution in normal pooled plasma [17]. This step is thought to minimize the variance in the resulting clotting time stemming from interindividual variation in plasma fibrinogen concentrations [16].

We are aware of only one *ex vivo* real-world paper that examined all the readily available screening coagulation assays [international normalized ratio (INR), activated partial thromboplastin time (aPTT) and TT] in relationship to patients treated with dabigatran [19], and none has examined plasma fibrinogen concentrations. We aimed to add to the existing published real-world experience with data we collected as part of an observational study. Furthermore, we aimed to test the hypothesis that some of the residual variability in the measured TT between patients can be explained by variability in plasma fibrinogen concentrations.

Methods

Study design

This was an observational study conducted in Christchurch, New Zealand from July 2012 to May 2013. The overarching goal was to assess real-world dabigatran pharmacokinetics and pharmacodynamics in relationship to renal function. Aspects of the data relevant to the aforementioned aims are presented here (other data and analyses from this study will be published elsewhere). Ethical approval for this study was obtained from the Upper South B Regional Ethics Committee, New Zealand (URB/12/02/009 and URB/12/02/009 AM01). Written consent was obtained from each individual who participated in the study.

Participants

Patients with AF who were ≥18 years old were included if they were on dabigatran etexilate at the same dose rate for ≥7 days and had not missed any doses in the 7 days prior to the study day (by their own report). Recorded details included demographics, dabigatran etexilate dose rates and thromboembolic and haemorrhagic risks according to published scoring systems [23, 24]. Estimated glomerular filtration rates were calculated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation [25]. Body surface area, calculated using Mosteller's equation [26], was used to convert the CKD-EPI result from units of millilitres per minute per 1.73 square metres to millilitres per minute.

Sample collection and laboratory analysis

Each recruited patient either provided two (2 and 10–16 h postdose) or five venous blood samples (1, 2, 4, 8 and 10–16 h postdose) in a single day. At each time point, both plasma dabigatran concentrations (BD Vacutainer® K_2 EDTA tubes) and clotting times (BD Vacutainer® citrate tubes) were measured. For each patient, a venous blood sample at one time point was collected to measure plasma creatinine concentrations (BD Vacutainer® lithium heparin tubes).

Dabigatran concentrations in human plasma were analysed by a validated liquid chromatography–tandem mass spectrometry method based on a previously published method [27]. Briefly, 50 µl of plasma was added to 450 µl of the internal standard, [$^{13}C_6$]-dabigatran (10 µg l^{-1} in methanol and 0.1 mmol/L aqueous HCl (9:1, v/v)). The mixture was vortexed and then centrifuged at 15 000***g*** for 5 min to precipitate the proteins. A 50 µl aliquot of clear supernatant was mixed with 500 µl of water, and 10 µl was injected into the liquid chromatography–tandem mass spectrometry system (Agilent 1290 Infinity Series High Performance Liquid Chromatograph connected to an Agilent 6460 Series Triple Quadrupole Mass Spectrometer; Agilent Technologies, Santa Clara, CA, USA). A Poroshell 120 EC C18 2.7 µm, 50 mm × 3.0 mm column (Agilent Technologies) was used for separation under gradient elution with acetonitrile increasing from 1 to 90% within 2 min in 0.2% formic acid and 10 mmol l^{-1} ammonium formate. The total analysis time was 5 min. Mass spectrometric detection was in the positive mode with dabigatran and [$^{13}C_6$]-dabigatran monitored at *m/z* 471.5→289.1 and *m/z* 477.5→295.1,

Table 1

Details of dabigatran and coagulation-related assays

Assay	Clot-activator reagent	Interday CV	Reference range*
Dabigatran	Not applicable	11.8% at 5.0 μg l^{-1} 4.3% at 50 μg l^{-1} 2.9% at 200 μg l^{-1} 2.9% at 1000 μg l^{-1}	Not applicable
INR	HemosIL Recombiplastin 2G	2.4% at 1.0 4.4% at 2.5	0.8–1.2
aPTT	Triniclot APTT HS	2.5% at 29 s 4.5% at 70 s	23–35 s
TT	HemosIL Thrombin Time	5.3% at 30 s	18–28 s
HTI	HTI α-thrombin	10.8% at 100 μg l^{-1} 4.7% at 280 μg l^{-1}	Not available
Fibrinogen	HemosIL Fibrinogen C	5.5% at 2.2 g l^{-1} 6.0% at 1.0 g l^{-1}	1.5–4.0 g l^{-1}

Abbreviations are as follows: aPTT, activated partial thromboplastin time; CV, coefficient of variation; HTI, Hemoclot® Thrombin Inhibitor; INR, international normalized ratio; TT, thrombin time. *Canterbury Health Laboratories values for normal adults.

respectively. For the range of 5–1000 μg l^{-1}, the interday precision [coefficient of variation (CV)] values were ≤11.8% (see Table 1) and bias was ≤8.3%. These performance characteristics were calculated from the results of quality control samples that were analysed during patient sample runs. All patient samples were analysed in triplicate.

Samples were analysed using the conventional screening assays, including the INR, aPTT and TT. Details of the reagents and precision of these assays are provided in Table 1. For the TT, our laboratory has a maximal reported time of 300 s. For the purpose of presentation in the correlation plots, these have been set to 300 s, but have not been included in the linear regression analyses involving TT. Additionally, samples were analysed using the HTI assay. Plasma fibrinogen concentrations were measured using the Clauss method [28]. All of these coagulation-related assays were performed on an ACL TOP 700 instrument (Instrumentation Laboratory, Bedford, MA, USA).

Serum creatinine was measured using an Abbott® Aeroset analyser (Abbott Park, IL, USA) by the modified Jaffe reaction. This was isotope dilution mass spectrometry (IDMS) aligned for the period of this study and had an interday CV of <4.0%.

All samples were analysed at Canterbury Health Laboratories (Christchurch, New Zealand). Apart from the dabigatran assay and the HTI, all other assays were the same as those employed in routine clinical work.

Statistical analysis

Statistical analysis was performed using GraphPad Prism (version 6.03; GraphPad Software, La Jolla, CA, USA; http://www.graphpad.com) and SPSS (IBM SPSS Statistics for Windows, version 20.0.0.2; IBM Corporation, Armonk, NY, USA). A value of $P < 0.05$ was considered statistically significant.

Linear regression analyses were used to determine the lines of best fit, and the coefficient of determination (r^2) was used to describe the relationships between the coagulation assays and plasma dabigatran concentrations. The exception was the aPTT, for which a square root regression model was employed, as per Stangier *et al*. [13]. We repeated the analyses involving aPTT and TT using ratios rather than the reported values. The ratios were calculated by dividing the reported values by the midrange value of the reference ranges for aPTT (23–35 s) and TT (18–28 s), which were 29 and 23 s, respectively. Linear regression analysis was also used to determine the line of best fit and the r^2 to describe the relationship between TT and plasma fibrinogen concentrations.

To discern the contribution of plasma fibrinogen concentrations to the variability in measured TT, the plasma fibrinogen concentrations were transformed into standardized values (z-scores) [29]. Furthermore, the standardized residuals from the linear regression of TT against plasma dabigatran concentrations were generated [29]. The standardized fibrinogen values and, separately, the standardized residuals, were tested for normality using the D'Agostino–Pearson omnibus test (with $P > 0.05$ indicating that the data passed the normality test). If normality was demonstrable, the standardized fibrinogen values were then linearly regressed against the standardized residuals. Finally, the r^2 of a linear regression model including both plasma dabigatran and fibrinogen concentrations against TT was generated.

Results

There were 52 individuals (age range of 38–94 years) recruited, whose characteristics are described in Table 2. Six individuals provided five samples, 44 provided two samples, and two provided one sample each (troughs only), for a total of 120 samples.

Table 2

Patient characteristics (n = 52)

Characteristic	Mean (SD)*
Age (years)	65 (12)
Male [*n* (%)]	41 (79)
Weight (kg)	98 (23)
Height (m)	1.75 (0.08)
BMI (kg m^{-2})	31.8 (7.1)
BSA (m^2)	2.17 (0.28)
Estimated GFR [ml min^{-1} (1.73 m^2)$^{-1}$]	72 (13)
Estimated GFR (ml min^{-1})	91 (23)
CHA_2DS_2-VASc	2.5 (1.7)
HAS-BLED	1.0 (1.0)
Dabigatran etexilate dose rate	
75 mg twice daily [*n* (%)]	3 (6)
110 mg twice daily [*n* (%)]	24 (46)
150 mg twice daily [*n* (%)]	25 (48)

Abbreviations are as follows: BMI, body mass index; BSA, body surface area; GFR, glomerular filtration rate; CHA_2DS_2-VASc and HAS-BLED are scoring systems for assessing thromboembolic and haemorrhagic risk, respectively, in the setting of atrial fibrillation [23, 24]. *Unless stated otherwise.

The correlation plots and r^2 values between the four coagulation assays and plasma dabigatran concentrations are shown in Figure 1 (plasma dabigatran concentrations 9–408 μg l^{-1}). For the analyses involving the aPTT and TT assays, the use of ratios instead of reported values did not alter the results (data not shown). Forty-five TT values were <300 s (plasma dabigatran concentrations 9–74 μg l^{-1}), while the remaining 75 values were >300 s (plasma dabigatran concentrations 61–408 μg l^{-1}).

The median (range) of the 120 plasma fibrinogen concentrations was 2.3 (1.1–3.9) g l^{-1}. The standardized fibrinogen values and standardized residuals (from the linear regression of plasma dabigatran concentrations against TT) both passed the normality test, with P = 0.09 and 0.10, respectively. All 45 samples with associated TT < 300 s were associated with plasma fibrinogen concentrations within the laboratory reference range, with a median (range) of 2.5 (1.7–3.9) g l^{-1}. Figure 2 shows the plots using these 45 plasma fibrinogen concentrations. We were unable to demonstrate a significant relationship between plasma fibrinogen concentrations and TT (Figure 2A). However, we found that plasma fibrinogen concentrations explained a small but significant part of the variability in the residuals from the linear regression of plasma dabigatran concentrations against TT, with r^2 = 0.12 (P = 0.019; Figure 2B). The linear regression model for the TT including plasma dabigatran and fibrinogen concentrations is shown in Table 3 (r^2 = 0.74).

Discussion

This observational study represents, to our knowledge, the largest single data set (in terms of samples analysed) of patients with AF prescribed dabigatran etexilate outside of phase I–III studies. Furthermore, we are the first to analyse the additional contribution, over and above plasma dabigatran concentrations, of plasma fibrinogen concentrations to explaining TT results in patients on dabigatran etexilate.

Our r^2 values, obtained by regressing the results from coagulation assays against plasma dabigatran concentrations measured using liquid chromatography–tandem mass spectrometry, were 0.49, 0.54, 0.70 and 0.95 for the INR, aPTT, TT and HTI, respectively. These compare with reported r^2 values from other *ex vivo* studies of 0.48–0.86 [13, 19, 22], 0.52–0.85 [13, 20, 22], 0.75–0.97 [13, 19] and 0.92–0.99 [7, 19, 20, 22] respectively. Together, these show that all of the screening coagulation assays have a significant relationship with plasma dabigatran concentrations.

For the clinician managing a patient treated with dabigatran etexilate, it is important that the coagulation assay being employed is at least capable of detecting the presence and effect of dabigatran over the range of dabigatran concentrations that are likely to be observed in clinical practice. Our correlation plots in Figure 1 are useful in this regard. These demonstrate that plasma dabigatran concentrations as high as 200 μg l^{-1} are associated with INR and aPTT values within the local reference ranges (reflecting apparently normal coagulation). Less than 10% of individuals given dabigatran etexilate in the RE-LY trial of dabigatran *vs.* warfarin for AF had trough concentrations in excess of 200 μg l^{-1} [30]. Hence, the INR and aPTT have been regarded as being relatively insensitive to plasma dabigatran concentrations, particularly in comparison to the TT and HTI assays [31]. In contrast, the TT is clearly very sensitive, with all TT values in our study being well above the local reference range, even at plasma dabigatran concentrations as low as 9 μg l^{-1}.

At the present time, in terms of the conventional screening assays, Hawes *et al.* have suggested that a combination of all three is used if laboratory coagulation monitoring is deemed necessary in real-world patients, where the HTI and dabigatran assays are less accessible [19]. In this setting, it has been suggested that TT would be useful to cover the lower, and the aPTT and INR to cover the higher, plasma dabigatran concentrations. This is contingent upon the widely disseminated notion that the TT is too sensitive to dabigatran and is unable to gauge the higher concentrations accurately. The TT assay we used consistently resulted in times greater than the maximal reported time at plasma dabigatran concentrations >74 μg l^{-1} in our study, while Hawes *et al.* reported that that this value was 138 μg l^{-1} with the TT assay they used [19]. In contrast, data from Stangier *et al.* demonstrate that the TT assay they used (Biomatic B10 coagulometer; Desaga, Wiesloch, Germany) could measure TT values for plasma dabigatran concentrations of 0 to ~400 μg l^{-1} [7, 13]. This range encompasses >90% of the plasma

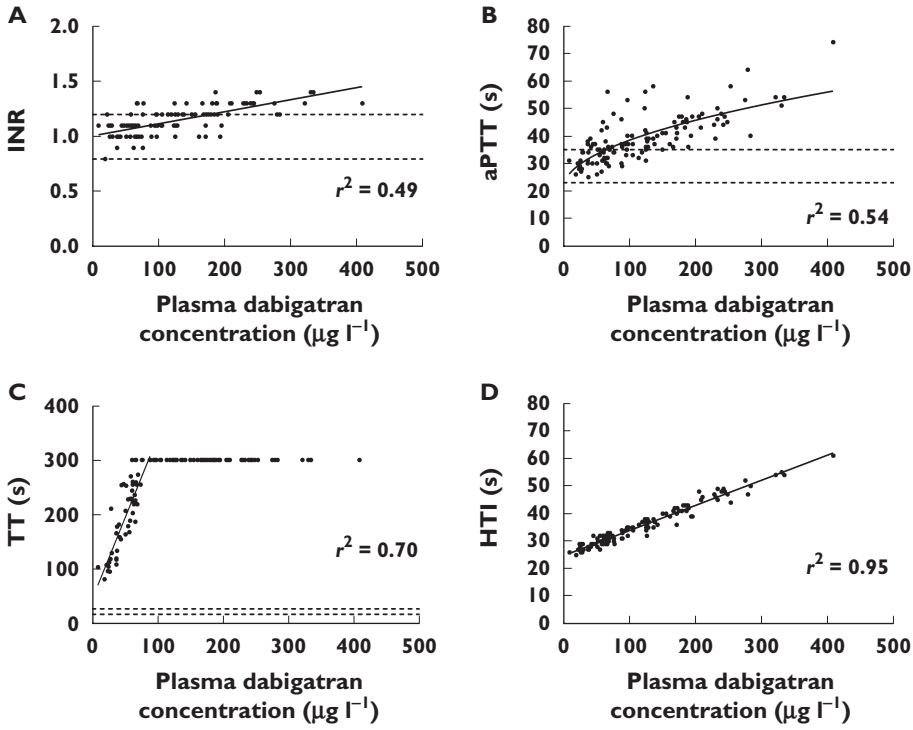

Figure 1

Correlation plots for 120 samples of international normalized ratio (INR; A), activated partial thromboplastin time (aPTT; B), thrombin time (TT; C) and Hemoclot® Thrombin Inhibitor (HTI; D) against plasma dabigatran concentrations. For TT, all values of TT >300 s have been set to 300 s. Continuous lines represent the lines of best fit. Dashed lines for (A), (B) and (C) are the references ranges at Canterbury Health Laboratories. The r^2 values are for the line of best fit, which for (C) does not include those data with TT > 300 s in the linear regression

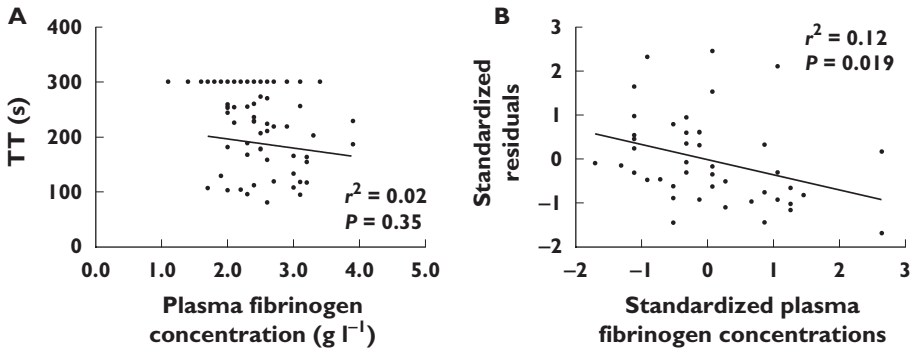

Figure 2

(A) Correlation plot of plasma fibrinogen concentrations against thrombin time (TT; for 120 samples, TT > 300 s have been set to 300 s). (B) Correlation plot of standardized residuals (from the linear regression of plasma dabigatran concentrations against TT) against standardized plasma fibrinogen concentrations (z-scores, for 45 samples with TT < 300 s). Lines of best fit are determined using the 45 samples with TT <300 s. The r^2 values are for the line of best fit, and *P* values are for the hypothesis that the slope of the linear regression is not zero

dabigatran concentrations reported in each of the real-world dabigatran studies, including the present study [19–22]. This should be explored further.

Douxfils *et al*. have previously reported that there is no significant correlation between plasma dabigatran and fibrinogen concentrations, which is in keeping with what might be expected biologically [16]. Given that one of the major differences between the HTI and TT assays is that the interindividual variability in plasma fibrinogen concentration is minimized in the HTI (r^2 = 0.95 against plasma dabigatran concentrations) compared with the TT (r^2 = 0.70), we hypothesized that including fibrinogen concentrations in the regression analysis would account for some of the explained variability of plasma dabigatran

Table 3

Linear regression model for thrombin time

Predictor	B	SE (B)	*P* value	r^2 change
Constant	100.0	26.7	0.001	
Plasma dabigatran concentration	3.0	0.3	<0.001	0.70
Plasma fibrinogen concentration	–22.0	9.1	0.02	0.04

Abbreviations are as follows: B, unstandardized coefficients; SE, standard error.

concentrations in relationship to the TT. While we demonstrated a statistically significant contribution to the explained variability of TT with plasma fibrinogen concentrations, the resulting $r^2 = 0.74$ for the combination of plasma fibrinogen and dabigatran concentrations for TT was only slightly greater than that observed with plasma dabigatran concentrations alone ($r^2 = 0.70$).

The apparent lack of proportional variance in the relationship between the HTI and plasma dabigatran concentrations is an interesting phenomenon (Figure 1D). This was also demonstrated by van Ryn *et al*. with a much larger number of samples encompassing plasma dabigatran concentrations up to 300 μg l^{-1}, albeit in healthy volunteers [7]. As discussed earlier, the dilution step in the HTI effectively renders the assay into a measure of all thrombin inhibitors in the test plasma, including dabigatran itself [17]. In contrast, the proportional variance that is expected in most assays [32] was demonstrated by van Ryn *et al*. in their report concerning the relationships between the screening coagulation assays (INR, aPTT and TT) and plasma dabigatran concentrations [7]. Furthermore, the dabigatran assay published by Delavenne *et al*., which we also used in this study, also displayed proportional variance [27].

Obesity was a significant feature of our participants (Table 2). This is relevant to consider in the context of this study. Total body weight is a key component in estimating creatinine clearance using the Cockcroft–Gault equation [33], which was found to be a strong determinant of dabigatran concentrations in the RE-LY trial [34]. Greater body weight is associated with higher estimated creatinine clearance, and hence lower dabigatran concentrations [30]. Furthermore, obesity is associated with increased plasminogen activator inhibitor 1 and plasma fibrinogen concentrations [35, 36]. Hence, obesity would be expected to be associated with increased thromboembolic risk in the setting of dabigatran therapy for AF [37]. As a *post hoc* analysis, we plotted body mass index against plasma fibrinogen concentrations for the samples used in the analyses against TT, and were unable to demonstrate a significant relationship [Pearson's *r* (95% confidence interval) = 0.14 (–0.16, 0.42), $P = 0.36$].

Our study has a number of limitations. Firstly, the relationships we examined for the coagulation-related assays are dependent upon the coagulometer and clot-activator reagent used [16, 19]. Nonetheless, as discussed, our results in terms of r^2 values are similar to what has been reported. Secondly, the INR was reported to one decimal place, as is routine at Canterbury Health Laboratories, and prothrombin time in seconds was not recorded, which would have provided more precision. This may have contributed to a value for r^2 less than that reported in the literature, as mentioned above. Thirdly, the unequal contribution to the 120 samples by the 52 patients may have biased the results. As a *post hoc* analysis, we re-examined the data, using only the 2 and 10–16 h samples (102 samples). The r^2 values for the coagulation assays were very similar to those reported above for the 120 samples, including INR ($r^2 = 0.44$), aPTT (0.55), TT (0.70, using the same 45 samples with TT < 300 s) and HTI (0.95). Fourthly, we measured only the plasma dabigatran concentration, without accounting for the dabigatran glucuronides, which are active metabolites of dabigatran [38, 39]. The glucuronides may be important, because the percentage contribution of the glucuronides to total active drug exposure ranges from 10 to 35% [13, 40]. The HTI is thought to account for all thrombin inhibition, irrespective of whether it is from dabigatran or its glucuronides [17]; therefore, the r^2 of 0.95 for HTI against plasma dabigatran concentrations in our study suggests that the percentage contribution from the glucuronides is relatively constant across the 120 samples analysed. Consequently, we do not believe that explicitly including the glucuronides in our plasma dabigatran concentrations would have significantly altered the relationships, in terms of r^2 values. Finally, some fibrinogen assays using the Clauss method, including the one we used, have been reported to suffer from interference from dabigatran, with higher dabigatran concentrations associated with factitiously reduced plasma fibrinogen concentrations [15, 41, 42]. The Clauss method employs the addition of a high thrombin concentration to the test plasma to incite clot formation; the time taken for this is compared with a calibration curve, from which the test fibrinogen concentration is determined. Hence, the presence of thrombin inhibitors is expected to prolong the time to clot formation, and thus falsely depress the measured fibrinogen concentration. Plasma dabigatran concentrations of 100 μg l^{-1} have been found to be associated with a 12% decrease in plasma fibrinogen concentrations when measured by the fibrinogen assay that we used [15, 41, 42]. This enables comparison with 74 μg l^{-1} (or around 100 μg l^{-1} if an additional 30% of the dabigatran glucuronides is included as an extreme estimate), which was the highest dabigatran concentration in the 45 samples we used for the fibrinogen *vs*. TT analyses. This interference would be expected to obscure the contribution of plasma fibrinogen concentrations to the variability of the TT and may explain the smaller than expected contribution we found of plasma fibrinogen concentrations for TT.

In conclusion, we have corroborated the published data on the relationship between coagulation assays and plasma dabigatran concentrations with our data set of real-world patients with AF. Furthermore, we have found a small but statistically significant contribution of plasma fibrinogen concentrations to TT in these patients. The TT is a widely available coagulation assay, and at least one version of this is capable of measuring TT values in plasma dabigatran concentrations encompassing >90% of the published concentrations observed in clinical practice. It will thus be useful for further work to examine the TT assays in patients on dabigatran to elucidate the causes of variance in the TT, including plasma fibrinogen concentrations. Furthermore, modifications of the TT assay that reduce its excessive sensitivity to dabigatran, while maintaining its sensitivity to intraindividual variance in fibrinogen concentrations, should be tested.

Competing Interests

All authors have completed the Unified Competing Interest form at http://www.icmje.org/coi_disclosure.pdf (available on request from the corresponding author) and declare: no support from any organization for the submitted work; no financial relationships with any organizations that might have an interest in the submitted work in the previous 3 years; no other relationships or activities that could appear to have influenced the submitted work.

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